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Compositions for liver regeneration and for the prevention of liver failure

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### **Field of the invention**

The present invention concerns the use of the human hepatocarcinoma-intestine-pancreas / pancreatic-associated protein (HIP/PAP) for stimulating liver regeneration and also for the prevention of liver failure.

### **Background art**

Liver failure occurs in a number of acute and chronic clinical conditions, including drug-induced hepatotoxicity, viral infections, vascular injury, autoimmune disease, or blunt trauma. In addition, patients subject to inborn errors of metabolism may be at risk for developing liver failure. Symptoms of liver failure occurring as a result of these clinical conditions include, for example, fulminant acute hepatitis, chronic hepatitis, or cirrhosis, and in many instances, the restoration of normal liver function is vital to the survival of patients. For example, cirrhosis is the seventh leading cause of death and the fourth disease related cause of death in people between the ages of 25 to 44. (Source: American Liver Foundation).

In acute liver disease, the liver is able to regenerate after being injured. If the disease progresses beyond the liver's capacity to regenerate new cells, the body's entire metabolism is severely affected. Loss of liver function may result in metabolic instability combined with disruption of essential bodily functions (i.e., energy supply, acid-base balance and thermoregulation.) If not rapidly reversed, complications such as uncontrolled bleeding and sepsis occur, and dependent organs such as the brain and kidneys cease to function because of toxic by-products or because the liver is no longer synthesizing important nutrients. After large liver damage, liver tissue loses its regenerative and metabolic functions, and liver transplantation is a therapeutic strategy commonly used. However, the clinical application of liver transplantation is limited by the availability of human hepatocytes, liver tissue and the number of liver cells that can be transplanted safely at one time. Moreover, latency before surgery and post-surgery complications

could be critical to counteract the acute phase of liver failure. Another therapeutic strategy consists in a liver resection (removal of a portion of the liver). The most typical indications for liver resection are a malignant tumor, a hepatocellular carcinoma or a proliferative biliary diseases including cholangiocarcinoma. Tumors can be primary (developed in the liver) or metastatic (developed in another organ, then migrated to the liver). The majority of liver metastases come from the colon. The single tumor or more than one tumor confined to either left or right side of the liver can be successfully resected with 5-year survival as high as 60%. Liver resections performed on patients with extrahepatic disease may relieve the symptoms caused by the tumor, but offer little improvement in survival. Benign tumors of the liver (adenoma, and focal nodular hyperplasia) can be successfully managed by liver resection as well. Liver resections are also performed on people willing to donate part of their liver.

Taking into account the importance of liver transplantation and liver resection, Several strategies have been suggested to stimulate liver regeneration and suppress or limiting liver failure in the case of liver resection or transplantation.

Liver cell is believed to be controlled by various growth stimulatory and growth inhibitory cytokines of autocrine or paracrine origin, however, the exact role and action mechanism of these growth factors is far from entirely understood. Cytokines are secreted peptides or proteins that regulate the intermediary metabolism of amino acid, proteins, carbohydrates, lipids and minerals. Cytokines include peptides or proteins that act to mediate inflammation and are involved in intracellular communication modulating cell proliferation, and adhesion of inflammatory cells to the walls of the vessels, and to the extra cellular matrix. Cytokines are essential for the communication between the liver and extrahepatic sites and within the liver itself. Cytokines interact with hormones such as glucocorticoids, resulting in a complex network of mutual control. Many cytokines exert growth activity in addition to their specific proinflammatory effects. The liver is an important site of cytokine synthesis and the major clearance organ for several cytokines. In liver disease, cytokines are involved in the onset of intrahepatic immune

responses, in liver regeneration, and in the fibrotic and cirrhotic transformation of the liver.

Liver cell is also believed to be controlled by various growth factors. Growth factors are required to regulate developmental events or required to regulate expression of genes encoding other secreted proteins that may participate in intracellular communication and coordination of development and includes, insulin-like growth factor-I and II (IGF I and II), epidermal growth factor (EGF), type a and type b transforming growth factor (TGF- $\alpha$  and TGF- $\beta$ ), platelet-derived growth factor (PDGF).

*In vitro*, DNA synthesis in isolated hepatocytes has been shown to be stimulated by growth factors such as TGF $\alpha$ , or EGF. A further protein, named hepatocyte growth factor (HGF) has been shown to be a mitogen for primary hepatocytes.

Based on these observations, It has been proposed that these factors may be important mediator of liver regeneration. Consequently, growth factors as TGF $\alpha$ , EGF or HGF with growth factor-like activities have been indicated in the treatment of liver regeneration. However, these therapeutic strategies, suggested to stimulate liver regeneration and suppress liver failure, have not proved their efficacy without toxicity, and adverse effects. Namely, these growth factor favor tumor progression (Gang-Hong, et al., 1992 ; Horiguchi, et al. 2002).

Consequently, there remains a need in the art for an effective method which would stimulate liver regeneration, would protect against liver failure, and would be deprived of adverse toxic and tumorigenic effects. This need exists in any patient population in which liver damage has been induced. This need exists not only for transplanted patients but also for donors, and patients having undergone a liver resection. Further, there is still a need in the art for novel therapeutically useful compounds, which stimulate liver regeneration.

Additionally, taking into account the poor availability of donor organs, living donor partial liver transplantation is recognized as a measure for overcoming the lack of organs, and facilities for partial liver transplantation. However, partial liver transplantation cannot be considered as a safe operation for adults representing the majority of

transplantation patients because the resectable liver weight of donors is often less than the necessary liver weight for recipients. Thus there is a need for a mean for safe and rapid liver regeneration for small grafts.

Accordingly, it is an object of the present invention to provide a mean for the stimulation of liver regeneration after partial resection. An object of the present invention is also to provide a drug that can promote liver regeneration or hepatocyte growth after liver transplantation such as partial liver transplantation, and also after the occurrence of a discare causing liver failure, such as cirrhosis, acute hepatitis and chronic hepatitis.

These and further objects will be apparent to one ordinary skill in the art.

#### Summary of the invention.

The present invention is based on the experimental finding that HIP/PAP has mitogenic and antiapoptotic effects *in vitro* on hepatocytes in primary culture. Moreover, HIP/PAP is a mitogenic molecule for hepatocytes, during liver regeneration, *in vivo*. The present invention is also based on the experimental finding that HIP/PAP has no adverse effects in mammals.

A first object of the invention consists in a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

In another aspect, the present invention relates to a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of the human hepatocarcinoma-intestine-pancreas/ pancreatic-associated protein (HIP/PAP) of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

the present invention also relates to a pharmaceutical composition with limited adverse effects on liver necrosis comprising :

- (i) a therapeutically effective amount of a hepatotoxic compound,
- (ii) a liver damage effective amount of a polypeptide as defined above.

### Description of drawings:

**Figure 1** Schematic representation of the transgene.

The enhancer (2 kb) and promoter (0.3 kb) of the regulatory regions of the mouse albumin gene are indicated by dotted lines. Exons II, III, IV, V and VI and introns of the human HIP/PAP gene (1.6 kb) are indicated by black boxes and a dotted line, respectively. The bovine growth hormone poly A fragment (1021-1235) pcDNA 3.1 is indicated by a dotted line. Plasmid DNA is indicated by the heavy line. Relevant restriction sites are indicated by the arrows.

**Figure 2** Immunodetection of HIP/PAP protein.

- A. Immunohistochemistry: original magnification x20, 1 wild-type liver, 2 HIP/PAP transgenic liver, 3 wild-type hepatocytes, 4 HIP/PAP hepatocytes.
- B. Western blot hybridised with HIP/PAP and actin antibodies showing a band with the 16 kDa and the 45 kDa expected size, respectively. Lane 1 purified HIP/PAP protein (10 ng), lanes 2, 3 and 4, wild-type liver; HIP/PAP transgenic liver 27 and 24 homozygous strains, respectively, lanes 5, 6 and 7 wild-type, HIP/PAP 27 and 24 hepatocytes after isolation, respectively.

**Figure 3** Time course of *in vivo* hepatic regeneration after partial hepatectomy.

- A. Immunodetection of BrU-positive nuclei, in wild-type (1,2,3,4) and HIP/PAP transgenic livers (5,6,7,8); 1 and 5 24 hours, 2 and 6 36 hours, 3 and 7 46 hours, 4 and 8 55 hours after hepatectomy.
- B. Each box plot comprises five horizontal lines displaying the 10<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, percentiles of a variable. All values for the variable above the 90<sup>th</sup> percentile and below the 10<sup>th</sup> percentile are plotted separately, so that the box plots are valuable in highlighting any

outliers. Wild-type mice ( $n = 9$ ), and HIP/PAP transgenic mice ( $n=10$ ) ( $p=0.0014$ ).

C. Liver weights were measured in normal non hepatectomized mice. The liver/body ratio of weight was calculated and expressed as the average percentage  $\pm$  SD. There was no difference in this ratio between the two groups ( $0.0460 \pm 0.0064$ ,  $n = 12$  and  $0.0489 \pm 0.0035$   $n = 16$  for wild-type and HIP/PAP transgenic mice, respectively). The average percentage recovery of normal liver weight ( $\pm$  sd) in wild type (O) and HIP/PAP mice (■) at various time points after partial hepatectomy shows stimulated recovery in the HIP/PAP transgenic mice (5 to 9 mice were hepatectomized at each time for each group) The difference was statistically significant at 48 hours ( $p < 0.001$ ), 60 hours ( $p < 0.003$ ) and 96 hours ( $p < 0.002$ ).

**Figure 4** DNA synthesis in wild-type and HIP/PAP transgenic hepatocytes.

A. Immunodetection of BrU-positive hepatocytes at 60 hours, wild-type (a), HIP/PAP (b) (original magnification  $\times 200$ ). The values shown are the mean  $\pm$  SD of independent cultures from 12 mice of each genotype.

B. time-course of DNA synthesis in hepatocytes stimulated by EGF ( $30 \text{ ng.ml}^{-1}$ ), wild-type (O), HIP/PAP (■).

C. DNA synthesis in cultured hepatocytes 60 hours after plating: Growth Factors (EGF  $30 \text{ ng ml}^{-1}$ , HIP/PAP  $40 \text{ ng ml}^{-1}$ ) were added after cell attachment. Forskolin was added for the last 16 hours. The data from 4 to 20 of experiments were presented as mean  $\pm$  SD (□) wild-type, (■) HIP/PAP.

**Figure 5** HIP/PAP inhibits  $\text{TNF-}\alpha$  + ActD-induced apoptosis in cultured primary hepatocytes .

A. Dose-dependent  $\text{TNF-}\alpha$  reduction in cell viability in wild-type (□) and HIP/PAP (■) transgenic hepatocytes. The data presented are the mean  $\pm$  s.e.m of independent cultures with four replicates from five mice of each genotype.

B. Hepatocytes were treated as indicated for 17 hours, wild-type ( $\square$ ), HIP/PAP ( $\blacksquare$ ). The histograms represent the mean values  $\pm$  s.e.m. of three separate experiments with four replicates.

C. Pyknotic nuclei of hepatocytes still attached were stained with Hoechst 33258 (magnification  $\times 400$ ). Arrows indicate features of apoptotic bodies organized in "rosettes" characteristic of the hepatocyte apoptosis induced by  $\text{TNF-}\alpha$ , wild-type (1,3,5) HIP/PAP (2,4,6) control cultures: no addition (1 and 2),  $\text{TNF-}\alpha$   $2 \text{ ng ml}^{-1}$  + ActD (3 and 4),  $\text{TNF-}\alpha$   $20 \text{ ng ml}^{-1}$  + ActD (5 and 6).

**Figure 6** Stimulation of liver regeneration in SCID mice transplanted with hepatocytes from wild-type or HIP/PAP transgenic mice.

A. Macroscopic evaluation of the livers of SCID mice, transplanted with hepatocytes from wild-type or HIP/PAP transgenic mice, and killed 7 days after hepatectomy.

B. Box plots of the liver weights of hepatocyte-transplanted SCID mice 7 days after hepatectomy. A significant difference was observed ( $p=0.0008$ ) between SCID transplanted with wild-type hepatocytes versus SCID transplanted with HIP/PAP hepatocytes, by using the Mann-Whitney test.

**Figure 7** Stimulation of liver regeneration in SCID mice by HIP/PAP protein.

Box plots of the liver weights of SCID mice intra-splenic injected 36 hours after partial hepatectomy with HIP/PAP protein ( $600 \text{ ng/mice}$ ) or phosphate buffer saline (PBS) ( $100 \mu\text{L}$ ). Mice were killed 7 days after hepatectomy. A significant difference was observed ( $p=0.0022$ ) between SCID injected with HIP/PAP protein versus SCID injected with PBS, by using the Mann-Whitney test.

### Detailed description of the invention :

The inventors have found according to the invention that HIP/PAP has mitogenic and antiapoptotic effects *in vitro* on hepatocytes in primary culture. Moreover, HIP/PAP is a mitogenic molecule for hepatocytes, during liver regeneration, *in vivo*.

The human hepatocarcinoma-intestine-pancreas /pancreatic-associated protein (HIP/PAP) gene was identified because of its increased expression in 25% of human hepatocellular carcinoma by using Northern blot analysis (Lasserre et al., 1992). It had been shown that HIP/PAP protein is detected in normal subjects in the intestine (Paneth and neuro-endocrine cells) and the pancreas (acinar pancreatic cells and islets of Langerhans). The HIP/PAP protein is also detected in some potential progenitor liver cells around portal area of normal liver (Christa et al., 1999). HIP/PAP is rapidly overexpressed during the acute phase of pancreatitis. It also acts as an adhesion molecule for rat hepatocytes and interacts with extracellular matrix proteins such as laminin-1 and fibronectin. This protein contains a putative signal peptide, and thus belongs to group VII of the C-type lectin family, according to Drickamer's classification and structural analysis (Abergel et al., 1999).

Now, the inventors have found that liver regeneration is stimulated, *in vivo*, in mice expressing the human HIP/PAP gene, after partial hepatectomy. Additionally, It has been found according to the invention that HIP/PAP has a mitogenic effect also *in vitro* in primary culture hepatocytes. In another aspect, it has also been found according to the invention that HIP/PAP has an anti-apoptotic effect against apoptosis induced by TNF- $\alpha$  combined with actinomycin D in primary culture hepatocytes. It has also been shown according to the invention that hepatocytes that recombinantly express HIP/PAP induce liver regeneration, when injected locally in partially liver-resected mice. The inventors have shown that the HIP/PAP protein, when injected to mice having undergone partial hepatectomy, induces liver regeneration. Taking these observations into account, the inventors have shown that the HIP/PAP protein provides effective mitogenic and anti-apoptotic effects, protects against liver failure, *in vivo*, has no adverse effects and

is particularly devoid of any carcinogenic effect, in contrast to the growth factors known in the art such as HGF, TGF $\alpha$  or EGF, as described above.

5 Taken together, these results demonstrate the therapeutic importance of HIP/PAP in liver regeneration. Thus, these experimental results have allowed the inventors to design pharmaceutical compositions for stimulating liver regeneration *in vivo* comprising an effective amount of the human hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP) of sequence SEQ ID N°1, in  
10 combination with at least one physiologically acceptable excipient.

A first object of the invention consists in a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the  
15 amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

The invention also concerns a pharmaceutical composition comprising a polypeptide fragment of HIP/PAP, which is effective for liver  
20 regeneration. This polypeptide of sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 consists of a biologically active portion of the HIP/PAP protein, which had previously been described as a Carbohydrate Recognition Domain (CRD) sequence (Christa et al. 1994).

25 In a first preferred embodiment, the pharmaceutical composition of the invention comprises a biologically active portion of HIP/PAP as described hereabove, which can be isolated from cell or tissue sources by an appropriate purification scheme using standard protein purification techniques.

30 In another preferred embodiment of said pharmaceutical composition the biologically active portion of HIP/PAP is produced by recombinant DNA techniques, such as described in the examples. According to a third preferred embodiment, the biologically active portion of HIP/PAP is synthesised chemically using standard peptide synthesis  
35 techniques.

An isolated or purified biologically active portion of HIP/PAP is substantially free of cellular material or other contamination proteins from the cell or tissue source from which HIP/PAP is derived, or substantially free from chemical precursors when chemically synthesised.

To determine the percent of identity of two amino acid sequences, the sequence are aligned for optimal comparison purposes. For example, gaps can be introduced in one or both of a first and a second amino acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes.

For optimal comparison purposes, the percent of identity of two amino acid sequences can be achieved with CLUSTAL W (version 1.82) with the following parameters: (1) CPU MODE = ClustalW mp; (2) ALIGNMENT = « full »; (3) OUTPUT FORMAT = « aln w/numbers »; (4) OUTPUT ORDER = « aligned »; (5) COLOR ALIGNMENT = « no »; (6) KTUP (word size) = « default »; (7) WINDOW LENGTH = « default »; (8) SCORE TYPE = « percent »; (9) TOPDIAG = « default »; (10) PAIRGAP = « default »; (11) PHYLOGENETIC TREE/TREE TYPE = « none »; (12) MATRIX = « default »; (13) GAP OPEN = « default »; (14) END GAPS = « default »; (15) GAP EXTENSION = « default »; (16) GAP DISTANCES = « default »; (17) TREE TYPE = « cladogram » et (18) TREE GRAP DISTANCES = « hide ».

Biologically active portions of HIP/PAP include peptides comprising amino acid sequences sufficiently homologous to the full length amino acid sequence of HIP/PAP of SEQ ID N°1, which include the same number of amino acids than the full length HIP/PAP, and exhibit at least the same biological activity than HIP/PAP.

Biologically active portions of HIP/PAP include further peptides comprising amino acid sequences sufficiently homologous to the full length amino acid sequence of HIP/PAP of SEQ ID N°1, which include more amino acids than the full length HIP/PAP, and exhibit at least the same biological activity than HIP/PAP.

By the "same biological activity", as applied to biologically active peptides homologous to HIP/PAP, it is herein intended peptides that induce in vivo liver regeneration with the same order of magnitude than the full length HIP/PAP; as it can be easily determined by the one skilled

in the art, for example by measuring BrdU incorporation by heparocytes, and measuring liver mass restoration as it is shown in Example 2.

As used herein the biologically active portion of HIP/PAP encompasses a polypeptide comprising an amino acid sequence having 90% of identity with the polypeptide of sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1. According to the invention a first amino acid sequence having at least 90% of identity with a second amino acid sequence, comprises at least 90 %, and preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of identity in amino acids with said second amino acid sequence.

Polypeptides according to the invention comprise also variants, such as the CRD sequence from different mammals, and for example from the bovine pancreatic thread protein (BPTP) or the pancreatic associated protein 1 (PAP1) from the rat, described by Orelle, et al.

In addition to naturally occurring allelic variants of the biologically active portion of HIP/PAP sequences that exist in mammals, the person skilled in the art will further appreciate that changes can be introduced by mutation into the nucleotide sequence of SEQ ID N°1, thereby leading to changes in the amino acid sequence of HIP/PAP without altering the biological activity of HIP/PAP.

In addition, substitutions of non-essential amino acid can be made in the sequences corresponding to HIP/PAP. A "non essential" amino acid residue is an amino acid residue that can be altered from the wild type sequence of HIP/PAP without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity.

A second object of the invention consists in a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising a polypeptide of sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

Another object of the invention is a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid

identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 27 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

5 A further object of the invention consists in a pharmaceutical composition according to claim 1 comprising an effective amount of the polypeptide consisting of the amino acid sequence starting at the amino acid residue 27 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable  
10 excipient.

Another object of the invention consists in a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of the human hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP), in combination with  
15 at least one physiologically acceptable excipient.

Without wishing to be bound to any particular theory, the inventors believe that the complete HIP/PAP protein of sequence SEQ ID N°1, i.e. a polypeptide comprising the CRD sequence, a signal peptide, and a pro-peptide, leads to a best folding of said protein, particularly when said  
20 protein is produced through DNA recombinant techniques in eukaryotic cells that have been transfected with an expression cassette encoding it. By the way, a correct folding of the therapeutically active HIP/PAP may lead to a best biological efficiency for the pharmaceutical composition comprising said protein, for liver regeneration compared to a composition  
25 comprising only a portion of the protein.

In a preferred embodiment, HIP/PAP has the amino acid sequence shown in SEQ ID N°1. In other embodiments, HIP/PAP is substantially identical to SEQ ID N°1 and retains the same biological activity, for liver regeneration, when compared to the protein of sequence  
30 SEQ ID N°1, but differs in amino acid sequence due to natural allelic variations or mutagenesis. Accordingly, in another embodiment HIP/PAP is a protein which comprises an amino acid sequence of at least about, 90 %, and preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identity with the amino acid sequence of SEQ ID N°1.

The invention also encompasses HIP/PAP chimeric or fusion proteins. As used herein, a chimeric protein or a fusion protein comprises the polypeptides cited above which are fused to a non-HIP/PAP polypeptide. Within the fusion protein, the HIP/PAP polypeptide and the non-HIP/PAP polypeptide are fused to each other. The non-HIP/PAP polypeptide can be fused to the N-terminus or to the C-terminus of the HIP/PAP polypeptide.

For example, in one embodiment, the fusion protein is a GST-HIP/PAP fusion protein in which the HIP/PAP sequence is fused to the C-terminus of the GST sequence. Such fusion proteins can facilitate the purification of recombinant HIP/PAP.

In all cases the fusion proteins of the invention possess the same biological activity as HIP/PAP of SEQ ID N°1.

Two different pathways trigger liver regeneration, one causes the replication of differentiated hepatocytes or biliary cells after partial hepatectomy or bile duct ligation (Fausto et al., 1994, Fausto et al., 2000). The second regenerative pathway is triggered after toxic injury, on massive necrosis or carcinogenesis, when the proliferation of hepatocytes or biliary cells is impaired or slowed by the injury (Factor VM et al., Petersen B et al., (1998), Akhurst B et al.). Under these conditions, it has been proposed « stem-like » cells proliferate and differentiate into hepatocytes and biliary epithelial cells, and then repopulate the liver. In rodents, the so-called oval cells represent a heterogeneous cellular compartment in which well-defined subpopulations have yet to be isolated. In humans, the oval cell compartments may participate in repopulating the liver after acute massive necrosis, and has also been identified in chronic liver diseases (Roskams T, et al., Sell S et al.). As used herein, The phrase « liver regeneration » concerns the process by which « stem-like » cells proliferate and differentiate into hepatocytes and biliary epithelial cells, and then repopulate the liver as well as hepatocyte and biliary cells replication.

The term "Biologically active amounts" concerns the amount of the composition according to the invention sufficient for treating the liver diseases associated with a decreased number of hepatocytes, in which liver regeneration conducted by HIP/PAP can restore hepatic function.

Liver regeneration conducted by HIP/PAP can be useful in several situations such as surgery, transplantation, diseases, and after hepatotoxic compounds exposure conducting to liver necrosis or partial liver necrosis.

Firstly, the pharmaceutical compositions according to the invention are suitable in the treatment of liver failure, consecutive to liver resection and liver transplantation. The pharmaceutical composition according to the invention can be administrated to the donor of a liver transplantation, to the receipt of such transplantation, to patients after a liver resection, in order to prevent the establishment or progress of liver failure by stimulating liver regeneration.

By stimulating liver regeneration, the compositions of the invention have other beneficial effects. Among them, we can cite the opportunity to make liver transplantation and partial liver transplantation with high effectiveness and also the opportunity to stimulate liver regeneration ex vivo, for example stimulate the growth of a liver transplant, liver epithelial cells, liver stem cells, or HIP/PAP genetically modified cells before transplantation.

The phrase "liver failure" is used herein in the broadest sense, and indicates any structural or functional injury resulting, directly or indirectly from a decreased number of liver epithelial cells i.e. hepatocytes and biliary cells.

The term "liver transplantation" has the common meaning in the art and includes partial and whole liver transplantation in which a liver of a donor is partially or wholly resected and partially or wholly transplanted into a recipient. Partial liver transplantation is classified by operation mode into orthotopic partial liver transplantation, heterotopic partial liver transplantation, and the like, and the present invention can be applied to any of them. In partial liver transplantation, a liver transplant or a partial liver transplant from a donor corresponding to about 30-50% of the normal liver volume of a recipient is typically transplanted as a graft into the recipient whose liver has been wholly resected. But the present invention has the effect of promoting liver regeneration or hepatocyte growth even if the graft is about 30% or less.

As used herein "liver transplant" means a liver transplanted into a recipient by the transplantation operation as described above, and also

includes the so-called "partial liver transplant" corresponding to a graft consisting of the part of the liver of a donor. Liver transplantation means also injection of hepatocytes (genetically modified or stimulated to proliferate or differentiate) into portal vein.

5 As used in the case of liver transplantation, the phrase "liver regeneration" means morphologic changes in which lost liver tissues are replaced by hepatocyte growth of a liver transplant or partial liver transplant, but also includes biochemical changes such as improvement, recovery, or normalisation of hepatic functions. Specific subjects to be  
10 treated by the composition of the invention includes, for example patients who received partial liver transplant after the liver had been wholly resected for treating hepatic failure caused by liver diseases such as hepatitis, hepatic cirrhosis of alcoholic, viral, drug or unknown cause, or hepatic cancer.

15 Compositions according to the invention are also suitable in the treatment of liver failure, consecutive to other diseases such as :  
Hepatitis B, Hepatitis C, Urea Cycle defects, Familial hypercholesterolemia, Alcohol induced cirrhosis, Glycogen Storage Disease, Autoimmune Hepatitis, Primary Hyperoxaluria type I,  
20 Cryptogenic cirrhosis, Crigler-Najjar syndrome type I, Congenital Hepatic Fibrosis, Neimann- Pick Disease, Primary Biliary Cirrhosis, Familial Amyloidosis, Biliary Atresia, Hepatocellular Carcinoma, Primary Sclerosing Cholangitis, Hepatoblastoma, Alagille Syndrome, Hemangioendothelioma, Familial Cholestasis, Non-Carcinoid neuro-  
25 endocrine, Drug induced liver failure, benign liver tumor such as focal nodular hyperplasia Liver tumors such as Hepatocellular carcinoma and Cholangiocarcinoma, Acute/fulminant liver failure, Budd-Chiari syndrome, Alpha-1-antitrypsin deficiency, Wilson Disease, Hemochromatosis, Tyrosinemia, Protoporphyrria, and Cystic fibrosis.

30 Compositions according to the invention are also suitable in the treatment of liver failure, consecutive to hepatotoxic compounds exposure conducting to liver necrosis or partial liver necrosis. A number of hepatotoxic compounds, including alcohol, virus, such as HBV, HCV or HIV, mushrooms, such as phaloide amanite, parasites such as  
35 Plasmodium Falciparum) or certain therapeutics, induce cytotoxicity and

liver necrosis. Among these therapeutics, we can disclose anaesthetics, such as Enflurane, neuropsychotropics such as Hydrazides, anticonvulsants such as valproic acid, analgesics, such as Acetaminophen, antimicrobials such as Amphotericin B or Penicillin, hormones such as Acetohexamides, cardiovascular drugs, such as Papaverine, Immunosuppressives and antineoplastics, such as asparaginase, anti-hypertension drugs, anti-inflammatory drugs and miscellaneous drugs such as vitamin A, Oxyphenisatin, iodide ion.

Although the exact mechanism of hepatotoxicity is uncertain, these compounds have deleterious effects on hepatocyte metabolism and contribute to the necrosis of hepatic tissue, and apparition of liver failure.

Thus, a further object of the invention consists in a kit with limited adverse effect on liver necrosis comprising :

- (i) a therapeutically effective amount of a hepatotoxic compound,
- (ii) an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

The invention also encompasses a kit comprising a polypeptide fragment of HIP/PAP, biologically active portion of HIP/PAP or the entire HIP/PAP protein as defined above. The hepatotoxic compound of the composition can be one of those cited above.

Although the following part of the specification relates especially to the formulation of compositions comprising the HIP/PAP protein, it is also suitable for therapeutic compositions comprising polypeptides fragments or biologically active portions of HIP/PAP.

For the purpose of the present invention, HIP/PAP can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby HIP/PAP is combined in admixture with a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Science, 16<sup>th</sup> ed., 1980, Mack publishing Co, edited by Oslo et al.

By « physiologically acceptable excipient » is meant solid or liquid filler, diluent or substance, which may be safely used in systemic or topical administration. Depending on the particular route of administration, a variety of pharmaceutically acceptable carriers well known in the art include solid or liquid fillers, diluents, hydrotopes, surface active agents, and encapsulating substances.

These compositions will typically contain an effective amount of the HIP/PAP protein, for example, from on the order of about 6 µg/ml to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient.

HIP/PAP may be administered parenterally or by other methods that ensure its delivery to the bloodstream in an effective form. HIP/PAP may preferably be administered using an intra-hepatic route. Dosages and desired drug concentrations of such pharmaceutical compositions may vary depending on the particular use envisioned. A typical effective dose in mouse experiments is about 30 µg/kg. Interspecies scaling of dosages can be performed in a manner known in the art., e.g. as disclosed in Mordenti et al., *Pharmaceut Res* 8 p1351 (1991).

The pH of the formulation depends mainly on the particular type and the concentration of HIP/PAP protein, but preferably ranges anywhere from about 3 to about 8.

Compositions particularly well suited for the clinical administration of HIP/PAP include sterile aqueous solutions or sterile hydratable powders such as lyophilised protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

Sterility is readily accomplished by sterile filtration through (0,2 micron) membranes.

The HIP/PAP protein pharmaceutical composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the

scheduling of administration, and other factors known to medical practitioners.

5 The therapeutically « effective amount » of HIP/PAP protein to be administered will be governed by such considerations, and is the minimum amount necessary to induce, or alternatively enhance liver regeneration and prevent liver failure. Such amount is preferably below the amount that is toxic to the mammal or renders the mammal significantly more susceptible to infections.

10 The term « administration » or « administered » as used herein in reference to HIP/PAP protein refers to that administration of HIP/PAP protein which occurs prior to, simultaneous with, or after a liver resection, or a liver transplantation.

### Cellular compositions according to the invention

15 An object of the invention is a composition comprising dividing hepatocytes in combination with a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

20 Another object of the invention is a composition comprising hepatocytes that have been transfected with an expression cassette that drives the expression of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

25 An expression cassette that drives the expression of a polypeptide as described above can be obtained for example as described in the part entitled "Transgenic mice expressing HIP/PAP in the liver".

30 The hepatocytes as used herein, are directly collected from a liver, or obtained from stem cells and particularly from bone marrow stem cells that have been differentiated into hepatocytes. The differentiation of bone-marrow stem cells in hepatocytes has been reported by Petersen *et al.*, (1999) and Mitchell *et al.* The recourse to such bone-marrow stem

cells can avoid recourse to hepatectomy for obtaining *in vitro* hepatocytes cultures.

A further object of the invention is a composition comprising an effective amount of bone-marrow stem cells in combination with a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

Without wishing to be bound to any particular theory, the inventors believe that the administration of bone marrow stem cells treated with HIP/PAP to a patient may accelerate the liver regeneration process.

The cellular compositions described above can be used for long-term *in vitro* culture of hepatocytes, for example for the purpose of *in vitro* cellular assays. The availability of the cellular compositions above avoid a recurrent recourse to hepatectomy for obtaining *in vitro* hepatocytes cultures. The invention also comprises pharmaceutical compositions for stimulating liver regeneration *in vivo* comprising an effective amount of a composition as defined here above.

In preferred embodiments of the present invention, the polypeptide from the compositions cited above is replaced by a polypeptide fragment of HIP/PAP, a biologically active portion of HIP/PAP or the entire HIP/PAP protein as defined in the present specification.

### **Process according to the invention**

Another object of the invention is a process for stimulating hepatocyte growth *in vitro* comprising :

- (a) collecting hepatocytes;
- (b) cultivating said hepatocytes in an appropriate culture medium ;
- (c) treating said hepatocytes with a mitogenic amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

By "mitogenic amount " it is meant that the hepatocytes will be treated with a sufficient amount of the polypeptide as defined herein before to induce hepatocytes growth when added into a culture of hepatocytes. Generally, a "mitogenic amount" as specified above consists of an amount of said polypeptide which induces proliferation of the cultured hepatocytes, as it can be easily determined by the one skilled in the art, for example through BrdU incorporation as disclosed in the examples.

Another object of the invention is a process for stimulating hepatocytes growth *in vitro* comprising :

- (a) collecting hepatocytes;
- (b) cultivating said hepatocytes in an appropriate culture medium ;
- (c) transfecting said hepatocytes with an expression cassette that drives the expression of the HIP/PAP protein in said hepatic cells.

Steps (a) to (c) can be conducted according to the techniques disclosed in example 5 and to the corresponding section in the part "material and methods".

The phrase "collecting hepatocytes", as used herein, means that hepatocytes are directly collected from a liver, or means that they are obtained from stem cells and particularly from bone marrow stem cells that have been differentiated into hepatocytes. The differentiation of bone-marrow stem cells in hepatocytes has been reported by Petersen *et al.*, (1999) and Mitchell *et al.* The recourse to such bone-marrow stem cells can avoid recourse to hepatectomy for obtaining *in vitro* hepatocytes cultures. Thus, another object of the invention is a process for stimulating hepatocyte growth *in vitro* comprising :

- (a) collecting bone marrow stem cells;
- (b) cultivating said bone marrow stem cells in an appropriate culture medium ;
- (c) treating said bone marrow stem cells with a mitogenic amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

Without wishing to be bound to any particular theory, the inventors believe that the treatment described above enhances the bone marrow stem cells ability to regenerate the liver. In preferred embodiments of the present invention, the polypeptide from the process cited above is replaced by a polypeptide fragment of HIP/PAP, a biologically active portion of HIP/PAP or the entire HIP/PAP protein as defined in the present specification.

#### **Use according to the invention**

Another object of the present invention consists of the use of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 in the manufacture of a pharmaceutical composition for stimulating liver regeneration *in vivo*.

A further object of the present invention consists of the use of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 in the manufacture of a pharmaceutical composition for the prevention of the establishment or progress of liver failure in a patient at risk for developing or having been diagnosed with liver failure.

The invention also encompasses the use of polypeptide fragments from HIP/PAP and biologically active portions of HIP/PAP as defined above.

In a preferred embodiment, the liver failure is a consequence of a liver resection, a liver transplantation, or hepatitis.

In a further aspect of the invention, the use according to the invention concerns a patient at risk for developing or having been diagnosed with a liver failure caused by a disease comprised in the group consisting of: Hepatitis B, Hepatitis C, Urea Cycle defects, Familial hypercholesterolemia, Alcohol induced cirrhosis, Glycogen Storage Disease, Autoimmune Hepatitis, Primary Hyperoxaluria type I,

Cryptogenic cirrhosis, Crigler-Najjar syndrome type I, Congenital Hepatic Fibrosis, Neimann- Pick Disease, Primary Biliary Cirrhosis, Familial Amyloidosis, Biliary Atresia, Hepatocellular Carcinoma, Primary Sclerosing Cholangitis, Hepatoblastoma, Alagille Syndrome, Hemangioendothelioma, Familial Cholestasis, Non-Carcinoid neuro-endocrine, Drug induced liver failure, benign liver tumor such as focal nodular hyperplasia Liver tumors such as Hepatocellular carcinoma and Cholangiocarcinoma, Acute/fulminant liver failure, Budd-Chiari syndrome, Alpha-1-antitrypsin deficiency, Wilson Disease, Hemochromatosis, Tyrosinemia, Protoporphyrria, and Cystic fibrosis

#### **Methods according to the invention**

Another object of the invention is a method for stimulating liver regeneration comprising administering an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 to a patient.

In a preferred embodiment, the method according to the invention encompasses a method comprising administering an effective amount of polypeptides fragments from HIP/PAP, biologically active portions of HIP/PAP or the entire HIP/PAP protein as defined in the present specification.

Another object of the invention is a method for the treatment of a patient with a hepatotoxic therapeutic agent effective in the prevention or treatment of a disorder or pathologic physiological conditions, comprising :

- (a) administering to said patient, simultaneously or in optional order, a biologically effective dose of said therapeutic agent and a preventatively effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

Another object of the invention is a method for the prevention of the establishment or progress of liver failure, consequence of a liver resection, a liver transplantation, or a hepatitis comprising administering to a patient an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

According to the method above, the polypeptide is administrated before, during or after a liver resection or a liver transplantation. The polypeptide can also be administrated to the donor of a liver transplantation, or to the receipt, in order to avoid for example post-surgery complications. According to the method above the polypeptide used is a fragment of HIP/PAP, a biologically active portion of HIP/PAP or the entire HIP/PAP protein as defined in the present specification.

Another object of the invention is a method for stimulating liver regeneration in a patient comprising:

- (a) collecting hepatocytes from said patient ;
- (b) cultivate said hepatocytes in an appropriate culture medium ;
- (c) treating said hepatocytes with a mitogenic amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 ; and
- (d) injecting said cells into said patient.

By "mitogenic amount " it is meant that the hepatocytes will be treated with a sufficient amount of the polypeptide as defined herein before to induce a liver regeneration when injected in a patient. Generally, a "mitogenic amount" as specified above consists of an amount of said polypeptide which induces proliferation of the cultured hepatocytes, as it can be easily determined by the one skilled in the art, for example through BrdU incorporation as disclosed in the examples.

Steps (a) to (d) can be conducted according to the techniques disclosed in example 5 and to the corresponding section in the part

"material and methods". In a preferred embodiment, the method comprises additional steps:

- (e) monitoring said patient for indication of liver failure, and
- (f) Continuing injections according to step (d) until said liver regeneration is sufficient.

Another object of the invention is a method for stimulating liver regeneration in a patient comprising:

- (a) collecting hepatocytes from said patient ;
- (b) cultivate said hepatocytes in an appropriate culture medium ;
- (c) transfecting said hepatocytes with an expression cassette that drives the expression of the HIP/PAP protein in said hepatic cells, and
- (d) injecting said cells into said patient.

An expression cassette that drives the expression of a polypeptide as described above can be obtained for example as described in the part entitled "Transgenic mice expressing HIP/PAP in the liver".

A further object of the invention is a method for stimulating liver regeneration in a patient comprising:

- (a) collecting bone marrow stem cells from said patient ;
- (b) cultivating said bone marrow stem cells in an appropriate culture medium
- (c) treating said cells with a mitogenic amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1
- (d) injecting the cells obtained at step (c) into said patient.

The availability of the method above avoid a recurrent recourse to hepatectomy for obtaining *in vitro* hepatocytes cultures. Without wishing to be bound to any particular theory, the inventors believe that the administration of bone marrow stem cells treated with HIP/PAP to a patient may accelerate the liver regeneration process.

Steps (a) to (d) can be conducted according to the techniques disclosed in example 5 and to the corresponding section in the part "material and methods". In a preferred embodiment, the method comprises additional steps:

- 5 (e) Monitoring said patient for indication of liver failure, and
- (f) Continuing injections according to step (e) until said liver regeneration is sufficient.

In another embodiment, the invention relates to a method for the prevention of the establishment or progress of liver failure in a patient at risk for developing or having been diagnosed with viral or autoimmune hepatitis, or a cirrhosis comprising administering to said patient a liver failure preventative amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1.

In another embodiment of the method above, the polypeptide used, is a fragment of HIP/PAP, a biologically active portion of HIP/PAP or the entire HIP/PAP protein as defined in the present specification.

The invention also concerns HIP/PAP, as an active ingredient of a composition for stimulating liver regeneration *in vivo*, comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1 in combination with at least one physiologically acceptable excipient.

Further details of the invention are illustrated in the following non-limiting examples.

### **MATERIALS AND METHODS**

#### **HIP/PAP production and purification :**

HIP/PAP was produced in transgenic mouse milk carrying the rabbit WAP gene able to drive expression of the HIP/PAP gene in the mammary gland, as previously described by the inventors (Christa et al., 2000).

Transgenic mice carrying the WAP/HIP construct were generated by microinjection into one-cell mouse zygotes of C57Bl/6xCBA hybrid strains. They were identified by tail DNA analysis on Southern blots. Mouse DNA was digested with *SacI*, and the generated fragments were separated on 1% agarose gels and transferred to Nytran 13N. The presence of the transgene was detected using a 4.4-kb *XhoI* fragment derived from the upstream region of the rabbit WAP gene.

All experiments, including animal welfare and conditions for animal handling before slaughter, were conducted in accordance with French Ministry of Agriculture guidelines (dated 19 April 1988).

#### - Milk samples

Milk was collected at day 13 postparturition from anaesthetised mice previously injected with 0.05 U of oxytocin to stimulate milk letdown. Mouse milk was diluted (1/10) in 10 mM Tris/HCl pH 7.5, 100 mM  $\text{CaCl}_2$ , and centrifuged for 30 min at 40 000 g. The pellet was discarded and the supernatant was spun again under the same conditions. The supernatant or lactoserum was used immediately for the purification of HIP/PAP or kept frozen at 20 °C.

#### - Purification of HIP/PAP protein from transgenic mouse milk

The resulting lactoserum (see above) was acidified to pH 4.6 by the addition of acetic acid (1 M) under stirring at 0 °C for 30 min. The precipitated material was removed by centrifugation at 110 000 g for 1 h in a Beckman 50.2 Ti rotor (Gagny, France). The supernatant was dialysed overnight at 4 °C against 1 L of 20 mM sodium acetate buffer pH 4.8, clarified by high speed centrifugation as above and filtered on a Millex 0.22 µm filter (Millipore, Guyancourt, France) before loading onto a Mono S HR 5/5 cation-exchange column previously equilibrated with 70 mM sodium acetate buffer pH 4.8. The flowthrough was discarded, and a 20-mL gradient of 0-500 mM NaCl in the working buffer was started when the absorbance returned to baseline. The column flow rate was 1 mL·min<sup>-1</sup>, and 1-mL fractions were collected. HIP/PAP-containing fractions were

pooled, diluted in 5 vol. of 140 mM sodium acetate buffer at pH 4.0 and reappplied to the Mono S HR 5/5 column equilibrated with 140 mM sodium acetate buffer pH 4.0. The flowthrough was discarded and the column was developed with a 20-mL gradient ranging from 0 to 400 mM NaCl in the working buffer. The column flow rate was 1 mL/min and 1-mL fractions were collected. Fractions containing HIP/PAP were pooled, diluted in 1 vol. of glycerol and stored at 20 °C.

Protein concentrations in the samples were determined using the Peterson protein assay. Denaturing polyacrylamide gels in sodium dodecyl sulfate (12.5% acrylamide, SDS/PAGE) were performed according to Laemmli. Coomassie blue staining gels were scanned and quantified using an imagemaster.

### Animals

#### - Transgenic mice expressing HIP/PAP in the liver.

The regulatory region of the mice albumin gene 18 was cloned upstream the HIP/PAP gene fragment to drive a human HIP/PAP gene expression specifically in the liver as described in the figure 1. The entire NotI/KpnI- linearized construct was microinjected into single cell mouse zygotes of hybrid strains in the Experimentation on the Transgenesis department (Villejuif France). The 24 and 27 homozygous transgenic lines were developed from independent founders on genetic background. Animal welfare, conditions for animal handling before slaughter and all experimental procedures were ensured in line with the French Ministry of Agriculture guidelines (dated 19<sup>th</sup>, April 1988).

#### - Control mice

C57BL/6 mice were provided by IFFA CREDO (L'Arbresle, France) and were used as controls of HIP/PAP transgenic mice.

#### - Recipients of isolated hepatocytes

Six-week-old female severe combined immunodeficient (SCID) mice (IFFA-CREDO, L'Arbresle France) were used as the recipients of hepatocytes isolated from male HIP/PAP transgenic mice or male C57BL/6 mice (IFFA-CREDO, L'Arbresle France), to minimize any risk of cell rejection.

#### **Partial Hepatectomy and BrdU Incorporation *in vivo*.**

Liver resection represents 70% of the total liver mass, as described by Higgins and Anderson (Higgins et al.) in two month old mice. Animals received one intra-peritoneal injection of 60 mg per kg body weight BrdU in 0.9% NaCl for 2 hours before dissection. They were sacrificed 24, 36, 46 and 55 hours post-hepatectomy. Animals and livers were weighted and BrdU-labelled nuclei were scored after incubation with anti-BrdU antibody (clone Bu 20A) and revelation was performed using the Universal LSAB2 horseradish peroxidase kit (Dako) with at least 20 low magnification ( $\times 10$ ) microscope fields for each liver slide (Olympus BX60). More than 1600 nuclei were screened per slide.

#### **Hepatocytes in primary culture**

Primary mouse hepatocytes were isolated from 2 to 3 months old mice, as previously described (Klaunig et al., Renton et al) with Liberase Blendzyme. Viable hepatocytes were purified using a low speed iso-density Percoll centrifugation method, as described by (Kreamer et al). Cells were resuspended in 199 medium containing penicillin, streptomycin, fungizone, bovine serum albumin (0,1%) and fetal calf serum (10%), at densities of  $2 \times 10^5$  and  $4 \times 10^5$  for proliferation and apoptotic experiments respectively in Primaria plates. Cells were maintained at 37°C in a humidified atmosphere and the medium was changed after attachment to the plates for 2 and 3 hours. Following attachment, the cells were rinsed once and cultured with the same medium containing no serum and then exposed to ActD ( $0,05 \mu\text{g} \cdot \text{ml}^{-1}$ ) plus TNF- $\alpha$  at ranges of concentration from 0,2 to 40 ng/ml for 17 to 18 hours, unless otherwise specified in the figure legends. For proliferation experiments, the medium was supplemented with 3,5,3'-triiodothyronine

5  $10^{-8}$  M, dexamethasone  $10^{-7}$  M, Insulin 10 µg/ml  $2 \times 10^{-6}$  M, transferrin 5,5 µg/ml, selenium 7 ng/ml, pyruvate 20 mM and foetal calf serum 5%.

#### **DNA synthesis in primary culture hepatocytes**

5

To measure DNA synthesis, BrdU (20 mM) was added for the last 16 hours prior to evaluation. The hepatocytes were washed with PBS, fixed, and rendered permeable in 30:70 acetic acid/ethanol solution at – 20°C for 30 minutes. Incorporated BrdU was localised using the BrdU  
10 Labelling and Detection kit II. Replicative DNA synthesis was measured by scoring the percentage of BrdU labelled cells in at least 10 low magnification microscope field for each sample (Olympus CK2). More than 1000 hepatocytes were screened per well.

15

#### **Cell viability and evaluation of apoptosis in primary culture hepatocytes.**

Seventeen hours after the addition of TNF- $\alpha$ , the monolayer was fixed with 4% paraformaldehyde for 20 minutes at room temperature, stained with Hoechst 33258 (0,5 µg/ml). Apoptotic cells were examined  
20 at wavelengths between 350 and 460 nm using an Olympus BX60 inverted fluorescence microscope (Olympus America Inc.). Loss of cell viability was quantified using the MTT assay: 30,000 cells per well in a 96 well microtiter plate were treated with x µl (0,5 mg/ml) MTT solution, freshly dissolved in medium for 1 hour at 37°C. The medium was then  
25 aspirated and 100 µl DMSO were added to solubilize the dye. Absorbance was measured at 570 nm using a Dynex MRX 96 well microplate reader (Dynex Technologies, France). Each measurement was performed in quadruplicate, for HIP/PAP and wild type hepatocytes dispensed on the same plate. Percentage cell survival was calculated by  
30 taking the optical density reading of cells receiving a particular treatment, dividing that number by the OD reading for untreated, control cells and then multiplying by 100. Comparison of the results with the number of apoptotic cells visualised using Hoechst 33258 validated the accuracy of the MTT assay.

35

### **Liver Cell isolation and Transplantation**

Hepatocytes were isolated from two-month old male HIP/PAP transgenic mice and male C57BL/6 mice, using the Liberase Blendzyme, as previously described by Klaunig and Renton. Viable hepatocytes were purified using a low-speed, iso-density Percoll centrifugation method, as described by Kreamer. Female SCID mice were anesthetized with xylazine (Bayer, Leverkusen, Germany) and ketamine (Biomérieux, Lyon France) dissolved in NaCl 0.9%, spleens were exteriorized through a small, left-flank incision, and a syringe with a 26-gauge needle was used to inject 100  $\mu$ l of cell suspension ( $0.75 \times 10^6$  viable hepatocytes) in Williams medium (Gibco/BRL). Recipient SCID was held for 30 days to allow sufficient time for the proliferation and reorganization of donor hepatocytes into the liver parenchyma, before partial hepatectomy was performed.

### **Evaluation of Liver Regeneration.**

Animals received one intra-peritoneal injection of 60 mg kg<sup>-1</sup> body weight BrdU in 0.9% NaCl 2 hours before dissection. They were sacrificed 24, 36, 46 and 55 hours post-hepatectomy. Animals and livers were weighted and BrdU-labelled nuclei were scored after incubation with anti-BrdU antibody (clone Bu 20A) and revelation was performed using the Universal LSAB2 horseradish peroxidase kit (Dako,) with at least 20 low-magnification (x10) microscope fields for each liver slide (Olympus BX60). More than 1600 nuclei were screened per slide.

### **HIP/PAP purified protein injection after Hepatectomy.**

Recombinant HIP/PAP protein was produced and purified as previously described (Christa et al., 2000), and was diluted in NaCl 0.9 % at 6  $\mu$ g/ml. 100  $\mu$ l HIP/PAP protein or PBS (Phosphate Buffered Saline) was injected into the spleens of Severe Cellular ImmunoDeficient (SCID) mice 36 h after partial hepatectomy. The animals were killed 8 days after partial hepatectomy.

### **Detection of Transplanted Liver Cells by RT-PCR analysis.**

RNA from frozen liver tissues was extracted according to TRIZOL reagent (Life Technologies) supplied instructions. CDNA was synthesised by 200 units Moloney murine leukaemia reverse transcriptase (Promega) and primed with 400 ng random primers (Invitrogen), from 1µg total RNA, at 42°C for 45 min, in the presence of 10 U RNasin, 1 x buffer supplied by the enzyme, 40 mmol l<sup>-1</sup> of the four deoxynucleotides. PCR was performed with 40 amplification cycles of 1 min each at the following temperatures : 94°C, 60°C, and 72°C, from 1/8 cDNA, by using pure Taq<sup>TM</sup> Ready -To-Go<sup>TM</sup> PCR Beads (Amersham Biosciences). Human HIP/PAP transgene expression was detected with primers 19/101. Endogenous HIP/PAP/Mo gene expression was detected with 104/105 primers which from the mouse published sequence of Itoh and Terakoa ().

19 sens : 5' cgc ccc ggg atg ctg cct ccc atg gcc ctg

101 antisens : 5' cgc gaa tcc gcc cat gat gag ttg cac acc aaa c 3'

104 sens : 5'cgc gga ttc atg ctg cct cca aca gcc tgc t 3'

105 antisens : 5' cgc aag ctt tta acc agt aaa ttt gca gac ata 3'

#### HIP/PAP assays : western blot analysis.

#### Immunohistochemistry and ELISA Test.

HIP/PAP protein was produced and purified from the milk of lactating transgenic mice as described above, and according to Christa et al., 2000. Western blot analysis and immunohistochemistry were performed with pre-HIP antibodies, as previously described (Christa et al., 1999). Serum HIP/PAP levels were assayed using a sandwich ELISA test, in accordance with the manufacturer's instructions (Dynabio, La Gaude, France).

#### Statistical analyses.

Results for hepatocytes in primary culture were expressed as mean +/- SD, and statistical significance (P<0.05) was determined using an unpaired Student's test. *In vivo* liver regeneration was represented by the percentages of nuclei incorporating BrdU using the box and whiskers

representation, and the statistical significance of differences between HIP/PAP transgenic and wild-type mice was determined by the Mann-Whitney U-test ( $P < 0.05$ ), because the data distribution was not normal (Statview 5', Abacus Concepts, Berkeley, CA).

## RESULTS

### Example 1: Characterization of human HIP/PAP transgenic mice.

The HIP/PAP transgene was specifically expressed in the liver, and HIP/PAP-expressing mice did not develop liver tumours, after a two year following. Immunohistolocalization analysis detected HIP/PAP protein in the liver of transgenic mice as diffuse intra-hepatocyte immunostaining, occupying most of the cytoplasm of the hepatocytes (figures 2A, 1 and 2). Staining was heterogeneous and positive regions were located either in centrilobular or portal areas of the liver acinus. This heterogeneous distribution likely reflects HIP/PAP secretion, thus hepatocytes could be either positive or negative before or after HIP/PAP secretion respectively. HIP/PAP protein was secreted into the serum (250 ng/ml to 700 ng/ml) in homozygote transgenic lines 24 and 27, and into the culture medium of primary hepatocytes (30 to 120 ng/ml per  $2 \cdot 10^5$  cells). No difference in morphology and ploidy was detected between HIP/PAP-expressing and control hepatocytes by histological examination (mouse hepatocytes were 80% binuclear after adhesion as previously described by Leist et al.). HIP/PAP immunohistochemistry views of hepatocytes in culture showed that more than 50% of the hepatocytes were HIP/PAP labelled (Figure 2A, 3 and 4). Western-blot analysis detected HIP/PAP as a 16 kDa protein in liver extracts and primary culture hepatocytes from HIP/PAP transgenic mice (figure 2B). HIP/PAP protein was not detected in wild type mice. Actin hybridization allowed an accurate estimation of the 50  $\mu$ g protein loaded for livers and hepatocytes (50  $\mu$ g corresponded approximately to 50,000 hepatocytes).

**Example 2 Liver regeneration is stimulated in mice expressing the human HIP/PAP gene.**

To test *in vivo* the HIP/PAP effect on liver cell proliferation, liver  
 5 regeneration induced by partial hepatectomy was examined. Low  
 magnification (x20) views for times 24, 36, 46 and 55 hours post partial  
 hepatectomy are presented Figure 3A. At the indicated times,  
 percentages of positive BrdU cells were higher in HIP/PAP transgenic  
 than in wild-type livers, despite the low overall frequency of nuclei which  
 10 had incorporated BrdU in both groups. The percentages of nuclei  
 incorporating BrdU were significantly higher in HIP/PAP transgenic mice  
 (median 33%; range 20-42%) compared to wild-type (median 18%; range  
 11-27%) ( $P = 0.0014$ ), 46 hours after partial hepatectomy (figure 3 B).  
 To reinforce the hypothesis that HIP/PAP protein may act as Growth  
 15 Factor during liver regeneration, the time-course of the hepatic mass  
 restoration in wild-type and transgenic mice was established, after  
 hepatectomy (figure 3C). Animal and liver weights were measured in  
 normal non hepatectomized mice. The liver/body ratio of weight was  
 calculated and expressed as the average percentage  $\pm$  SD. There was  
 20 no difference in this ratio between the two groups:  $0.0460 \pm 0.0064$ ,  $n=12$   
 and  $0.0489 \pm 0.0035$   $n = 16$  for wild-type and HIP/PAP transgenic mice,  
 respectively. Liver recovery was higher in the HIP/PAP transgenic than in  
 wild-type mice, and the difference was statistically significant at 48 hours  
 ( $p < 0.001$ ), 60 hours ( $p < 0.003$ ) and 96 hours ( $p < 0.002$ ). At 120 hours,  
 25 the liver weight recovered to the same percentage in both wild-type and  
 HIP/PAP transgenic mice.

**Example 3 HIP/PAP mitogenic effect in primary culture  
 hepatocytes**

30

In order to further investigate the enhanced liver regeneration  
 observed *in vivo* after hepatectomy in HIP/PAP transgenic mice, primary  
 cultures of hepatocytes were used to evaluate a HIP/PAP mitogenic  
 35 effect. Hepatocytes derived from HIP/PAP transgenic and wild-type mice

exhibited two peaks DNA synthesis, 60 and 84 hours after plating, when stimulated by EGF (figures 4 A and B). At 60 hours, mean percentages of BrdU-positive hepatocytes were  $31 \pm 7\%$  ( $n=19$ ) and  $16 \pm 4\%$  ( $n = 20$ ) in transgenic and wild-type mice, respectively ( $p < 0.0001$ ). When cells were stimulated by HGF, DNA synthesis was also higher in HIP/PAP than in wild-type hepatocytes ( $41 \pm 14\%$   $n = 4$ , versus  $31 \pm 11\%$ ,  $n = 4$ , respectively after 60 hours) although this difference did not attain significance. When hepatocytes were not stimulated by Growth Factor, BrdU incorporation were  $11\% \pm 3$  ( $n = 8$ ) and  $6\% \pm 3$  ( $n = 7$ ) in transgenic and wild-type hepatocytes respectively and the difference was statistically significant ( $p = 0.0146$ ). HIP/PAP is a secreted protein, and it was therefore tested whether it might act as a paracrine mitogenic factor. When HIP/PAP protein ( $40 \text{ ng} \cdot \text{ml}^{-1}$ ) was added to wild-type hepatocytes, EGF-induced DNA synthesis increased from  $16 \pm 4\%$  to  $24 \pm 7\%$  ( $p = 0.0168$ ;  $n = 8$ ; figure 4C). These results showed that HIP/PAP was a mitogenic factor for hepatocytes in primary culture. The mitogenic effect of HIP/PAP on hepatocyte proliferation was thus demonstrated both *in vivo* and *in vitro*.

#### Example 4 HIP/PAP anti-apoptotic effect against apoptosis induced by $\text{TNF-}\alpha$ + ActD in primary culture hepatocytes.

It was next examined whether the HIP/PAP mitogenic effect was associated with a HIP/PAP antiapoptotic effect. Rat hepatocytes in primary cultures were not sensitive to cell death caused by  $\text{TNF-}\alpha$  treatment alone. Instead, they die through apoptosis after exposure to  $\text{TNF-}\alpha$  combined with a low dose of ActD (22). Mouse hepatocyte cell death was induced by  $\text{TNF-}\alpha$  combined with an ActD dose as low as  $0.05 \mu\text{g}/\text{ml}$ , despite ActD ( $0.05 \mu\text{g} \cdot \text{ml}^{-1}$ ) alone did not induce any loss of viability (Figure 5B). It is shown (figure 5A) that hepatocytes expressing HIP/PAP resisted  $\text{TNF-}\alpha$  + ActD - induced apoptosis after a 16-17 hours of treatment. Cell survival reached 75% versus 43% ( $p < 0.0001$ ) for  $2 \text{ ng} \cdot \text{ml}^{-1}$   $\text{TNF-}\alpha$ , and 60% versus 27% for  $20 \text{ ng} \cdot \text{ml}^{-1}$   $\text{TNF-}\alpha$ , ( $p < 0.0001$ ). The  $\text{LD}_{50}$  for  $\text{TNF-}\alpha$  was over  $40 \text{ ng} \cdot \text{ml}^{-1}$  and  $1 \text{ ng} \cdot \text{ml}^{-1}$  in HIP/PAP and wild-type hepatocytes, respectively. Pre-treatment of cells with pan-

ant caspase z-VAD-fmk (50 $\mu$ M) completely prevented TNF- $\alpha$ -induced cell death, thus indicating that this process occurs via hepatocyte apoptosis. It was also examined whether dying cells exhibited the typical features of apoptosis. When stained with Hoechst 33258, non-viable cells displayed condensed chromatin, fragmented nuclei and apoptotic bodies, whereas viable cells did not. The features of apoptotic bodies were organized in "rosettes" characteristic of the hepatocyte apoptosis induced by TNF- $\alpha$  (Figure 5C). When HIP/PAP protein (40 ng ml<sup>-1</sup>) was added to wild-type hepatocytes, protection against 20 ng ml<sup>-1</sup> TNF- $\alpha$  + ActD rose from 27% to 47% (p<0.0001). These data demonstrate that HIP/PAP partly abrogated TNF- $\alpha$ -induced apoptosis in primary hepatocytes.

**Example 5 Liver regeneration is stimulated in mice by hepatocytes isolated from HIP/PAP transgenic mice.**

An in vivo experimental model was set up to test for the effect on overall liver regeneration of HIP/PAP expression in a minority of liver cells. Liver cell transplantation of hepatocytes isolated from HIP/PAP transgenic and C57BL/6 mice was thus performed, and then the extent of liver regeneration after partial hepatectomy was tested in the SCID recipient mice.

Two complementary approaches were used to assess liver cell transplantation. First, advantage of the human HIP/PAP transgene expression was taken to monitor the fate of transplanted liver cells, by using immunohistochemistry with HIP/PAP antibodies. A semi quantitative estimation indicated that transplanted cells constituted less than 1/1000 in the recipient livers. Moreover, HIP/PAP expression was shown in a limited number of liver cells without preferential distribution in the liver sections (portal or centrilobular area). Second, it was taken advantage of the presence of the human HIP/PAP sequence to perform RT-PCR. Human HIP/PAP expression was indeed detected in recipient SCID liver, before partial hepatectomy, thus confirming liver cell

transplantation Moreover; human HIP/PAP expression persists in the liver section obtained at different times after hepatectomy. These results demonstrated that transplanted cells persisted upon stimulation of recipient liver regeneration and retained gene expression.

5       The effects of the intrahepatic implantation of liver cells concerning the extent of liver regeneration after partial hepatectomy were then assessed. Macroscopic evaluation of the liver 8 days after partial hepatectomy showed a marked increase in liver mass recipient mice transplanted with liver cells from transgenic HIP/PAP mice (Figure 6A).  
10   Moreover, these findings were confirmed by liver weight measurements, which were significantly higher in recipient mice transplanted with liver cells from transgenic HIP/PAP (Figure 6B). BrdU incorporation analysis performed 48 h after partial hepatectomy did confirm a marked increase in cellular DNA synthesis upon transplantation of human HIP/PAP-  
15   expressing hepatocytes. Thus the transplantation of 750 000 viable hepatocytes was sufficient to increase liver regeneration. Standard histological examination of the liver did not reveal any obvious morphological changes.

#### 20   Example 6 Liver regeneration is stimulated in mice by administration of HIP/PAP

It was tested whether the injection of purified HIP/PAP might have the same effect as transplantation on liver regeneration. The weights of  
25   remnant livers were compared 8 days after partial hepatectomy in SCID mice that had been injected 36 h after partial hepatectomy with 100µl of purified HIP/PAP (600ng per mice). The results presented on figure 7 indicated a 10% increasing of liver weight in mice injected with HIP/PAP compared with that seen in mice injected with PBS. This observation  
30   demonstrated a mitogenic paracrine effect of HIP/PAP protein *in vivo*.

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### Claims

1. A pharmaceutical composition for stimulating liver regeneration *in vivo*  
5 comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically  
10 acceptable excipient.
2. A pharmaceutical composition according to claim 1 comprising an effective amount of the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the  
15 amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.
3. A pharmaceutical composition according to claim 1 comprising an effective amount of a polypeptide comprising an amino acid sequence  
20 having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 27 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.
- 25 4. A pharmaceutical composition according to claim 1 comprising an effective amount of the polypeptide consisting of the amino acid sequence starting at the amino acid residue 27 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.  
30
5. A pharmaceutical composition according to claim 1 comprising an effective amount of the human hepatocarcinoma-intestine-pancreas /pancreatic-associated protein (HIP/PAP) of sequence SEQ ID N°1 in combination with at least one physiologically acceptable excipient.  
35

6. A pharmaceutical composition according to any one of claims 1 to 5, for treating patients having undergone a liver resection.
7. A pharmaceutical composition according to any one of claims 1 to 5, for treating patients which are the subjects of a partial liver transplant, a hepatic failure caused by liver diseases such as hepatitis, hepatic cirrhosis of alcoholic, viral, drug or unknown cause, or hepatic cancer.
8. A pharmaceutical composition according to any one of claims 1 to 5, for treating patients which are the subjects of a disease comprised in the group consisting of : Hepatitis B, Hepatitis C, Urea Cycle defects, Familial hypercholesterolemia, Alcohol induced cirrhosis, Glycogen Storage Disease, Autoimmune Hepatitis, Primary Hyperoxaluria type I, Cryptogenic cirrhosis, Crigler-Najjar syndrome type I, Congenital Hepatic Fibrosis, Neimann- Pick Disease, Primary Biliary Cirrhosis, Familial Amyloidosis, Biliary Atresia, Hepatocellular Carcinoma, Primary Sclerosing Cholangitis, Hepatoblastoma, Alagille Syndrome, Hemangioendothelioma, Familial Cholestasis, Non-Carcinoid neuroendocrine, Drug induced liver failure, benign liver tumor such as focal nodular hyperplasia Liver tumors such as Hepatocellular carcinoma and Cholangiocarcinoma, Acute/fulminant liver failure, Budd-Chiari syndrome, Alpha-1-antitrypsin deficiency, Wilson Disease, Hemochromatosis, Tyrosinemia, Protoporphyrria, and Cystic fibrosis.
9. A pharmaceutical composition with limited adverse effects on liver necrosis comprising :
  - (i) a therapeutically effective amount of a hepatotoxic compound, and
  - (ii) an effective amount of a polypeptide according to anyone of claims 1 to 5.
10. A composition comprising dividing hepatocytes in combination with a polypeptide according to anyone of claims 1 to 5.
11. A composition comprising hepatocytes that have been transfected with an expression cassette that drives the expression of a

polypeptide according to anyone of claims 1 to 5 in said transfected hepatocytes.

- 5 12. A composition comprising an effective amount of bone-marrow stem cells in combination with a polypeptide according to anyone of claims 1 to 5.
- 10 13. A pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of a composition according to anyone of claims 10 to 12.
- 15 14. A process for stimulating hepatocyte growth *in vitro* comprising :  
(a) collecting hepatocytes;  
(b) cultivating said hepatocytes in an appropriate culture medium ;  
and  
(c) treating said hepatocytes with a mitogenic amount of a polypeptide according to anyone of claims 1 to 5.
- 20 15. A process for stimulating hepatocyte growth *in vitro* comprising :  
(a) collecting hepatocytes;  
(b) cultivating said hepatocytes in an appropriate culture medium ;  
and  
(c) transfecting said hepatocytes with an expression cassette that drives the expression of the HIP/PAP protein in said transfected hepatocytes.
- 25 16. A process for treating bone marrow stem cells *in vitro* comprising :  
(a) collecting bone marrow stem cells;  
(b) cultivating said bone marrow stem cells in an appropriate culture medium ; and  
30 (c) treating said bone marrow stem cells with a mitogenic amount of a polypeptide according to anyone of claims 1 to 5.

17. Use of a polypeptide according to anyone of claim 1 to 5, to manufacture a pharmaceutical composition for stimulating liver regeneration *in vivo*.

**ABSTRACT OF DISCLOSURE**  
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**Compositions for liver regeneration and for the prevention of liver failure.**  
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**INSTITUT NATIONAL DE LA SANTE ET DE LA  
RECHERCHE MEDICALE INSERM**  
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This invention is based on the experimental finding that HIP/PAP has mitogenic and antiapoptotic effects *in vitro* on hepatocytes in primary culture. The present invention is also based on the experimental finding that HIP/PAP administration has no adverse effects in mammals. This invention concerns a pharmaceutical composition for stimulating liver regeneration *in vivo* comprising an effective amount of a polypeptide comprising an amino acid sequence having 90% amino acid identity with the polypeptide consisting of the amino acid sequence starting at the amino acid residue 36 and ending at the amino acid residue 175 of sequence SEQ ID N°1, in combination with at least one physiologically acceptable excipient.

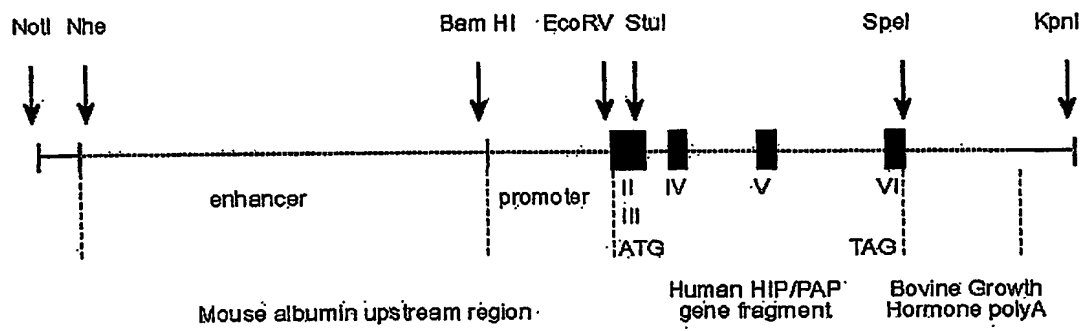


FIGURE 1

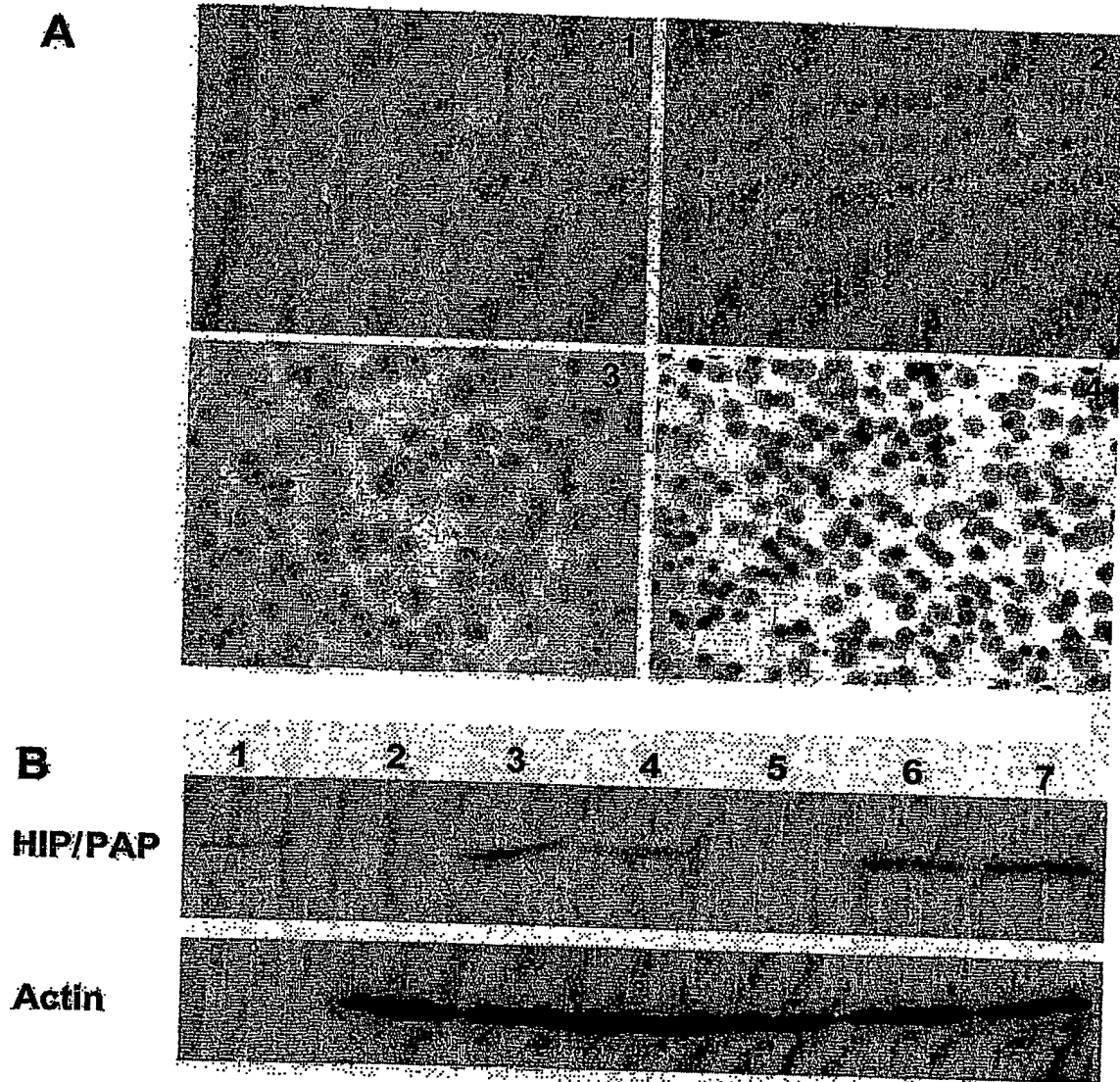


FIGURE 2

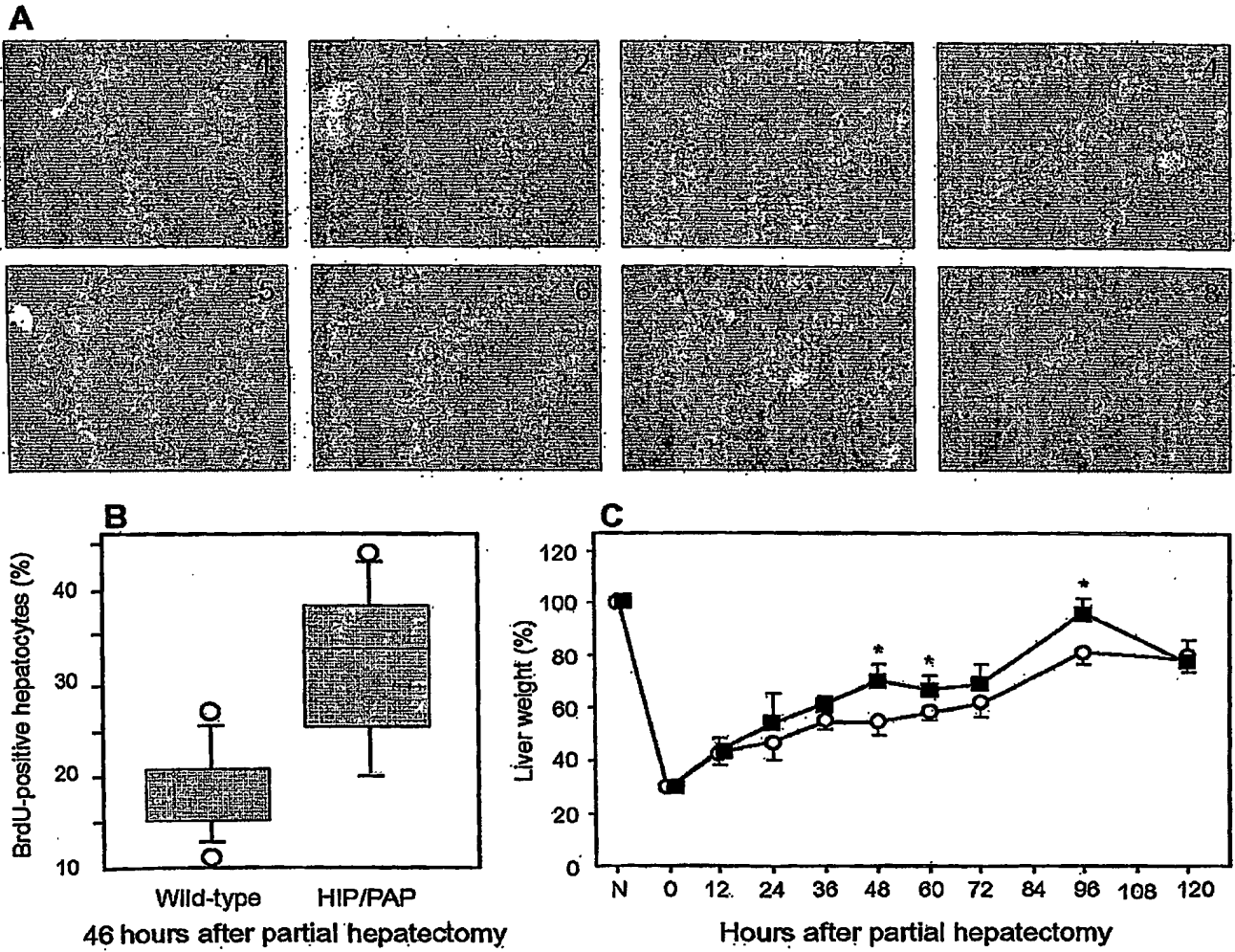
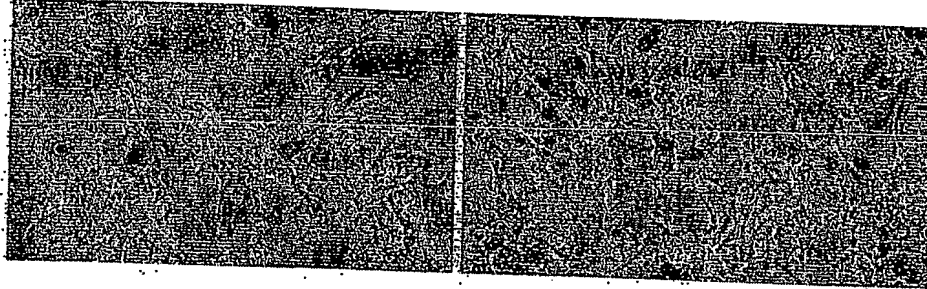
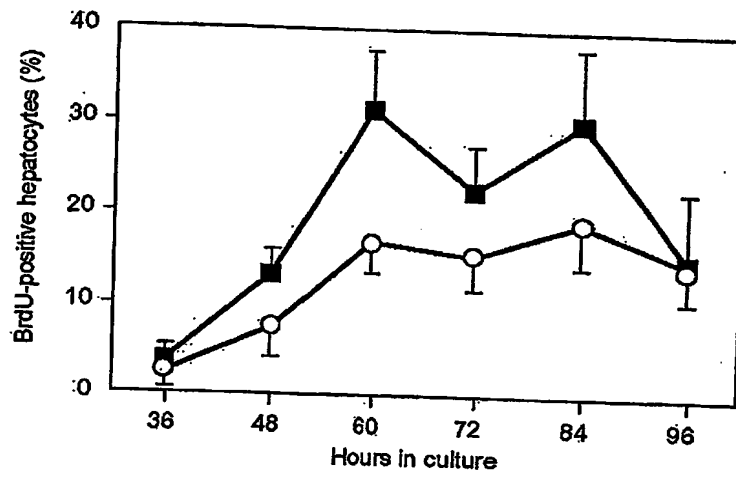


FIGURE 3

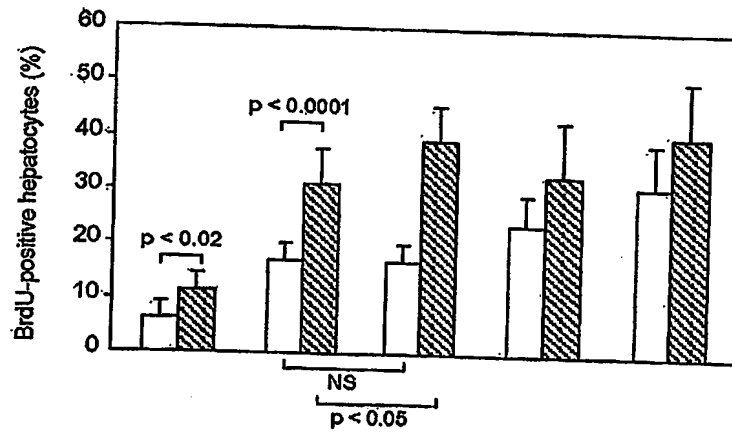
A



B



C



EGF (ng.ml <sup>-1</sup> )	0	30	30	30	0
FK (μM)	0	0	20	0	0
HIP/PAP (ng.ml <sup>-1</sup> )	0	0	0	40	0
HGF (ng.ml <sup>-1</sup> )	0	0	0	0	20

FIGURE 4

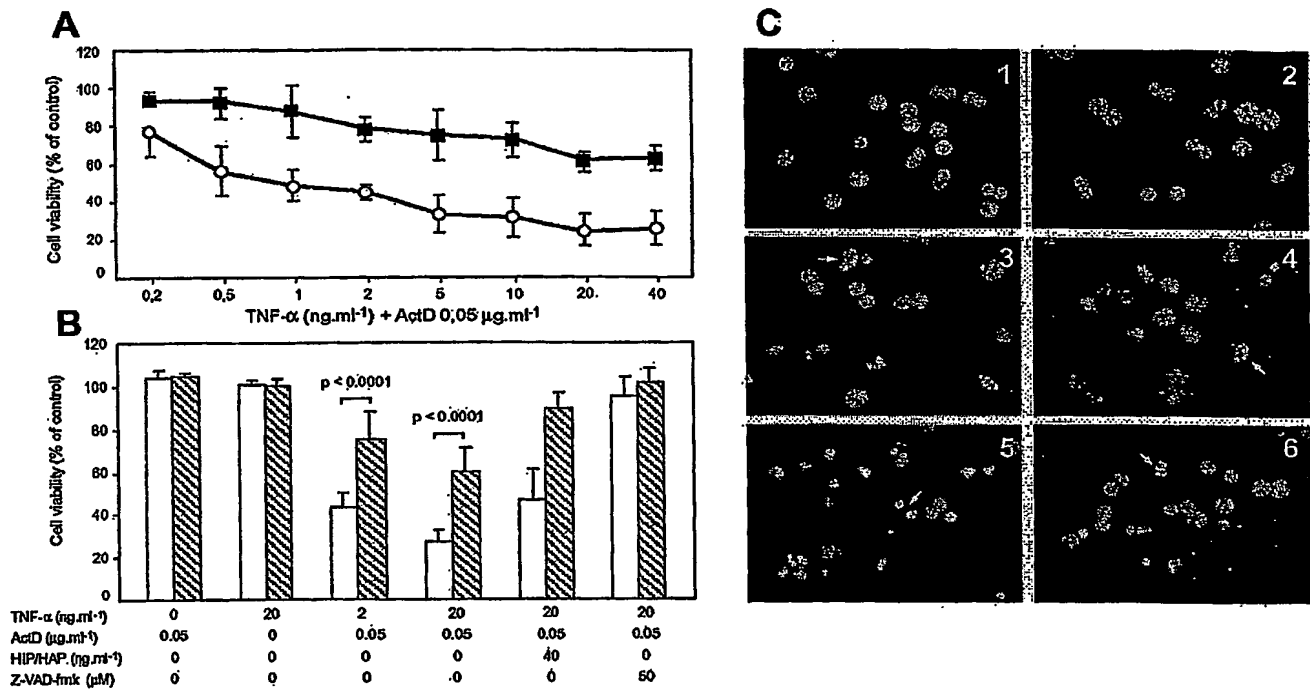


FIGURE 5

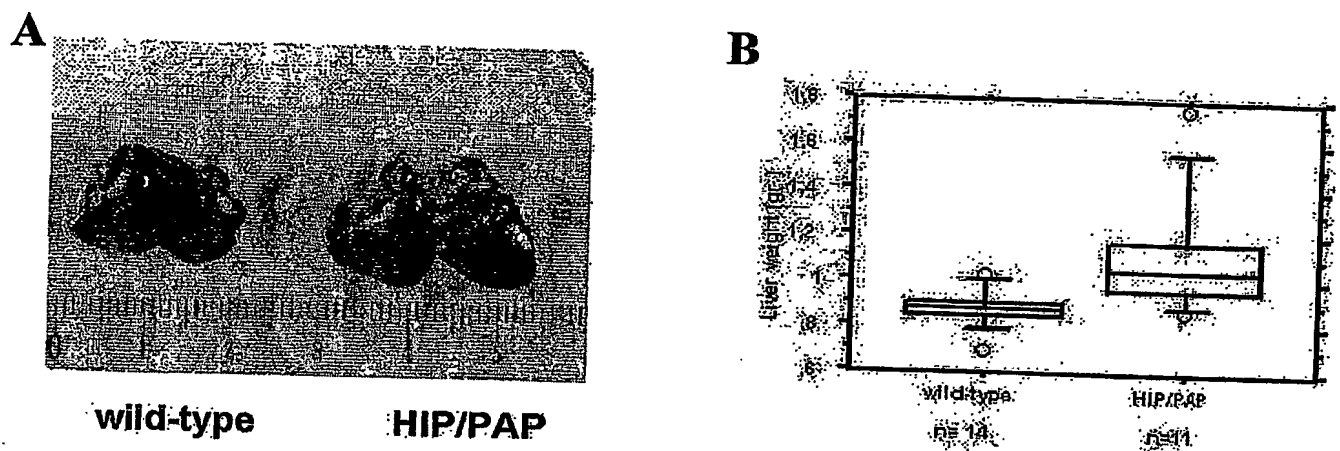
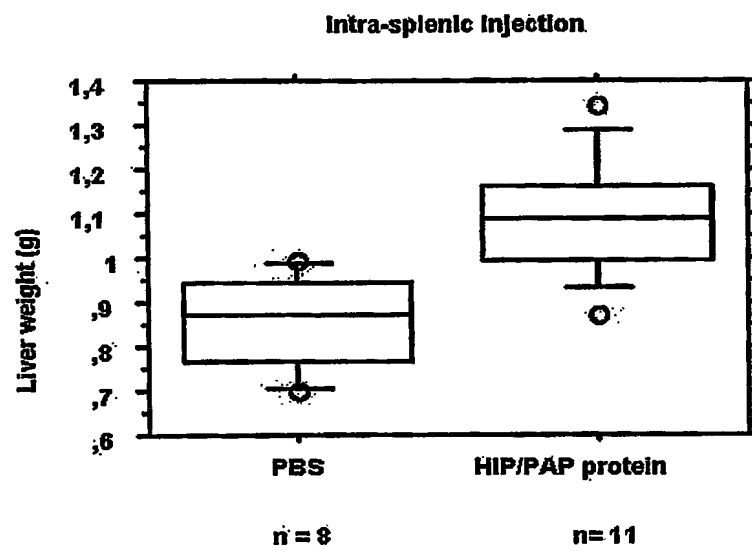


FIGURE 6

**FIGURE 7**

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